A HISTOCHEMICAL STUDY OF GLYCOGEN IN TISSUES OF THE ALBINO MOUSE BY USE OF THE FREEZING TECHNIQUE

A THESIS

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INTRODUCTION

This investigation was designed to study the glycogen content in the tissues of the albino mouse by the use of the freezing technique. Animals used in such a study should be provided with the same kind of diet for an accurate determination of the qualitative and quantitative distribution of glycogen in the various tissues. Functional differences in tissues should be indicated by the histochemical pictures of glycogen. Mann ('28) has shown that under wide variation of bodily activity the cytological picture of the liver is an ever changing one due to the function of the liver in the maintenance of blood sugar level. Accordingly, the basic activities in which other tissues participate may alter the distribution of substances within their cells.

Preparation of the tissues for study and also interpretation of results are of the utmost importance due to the occurrence of artifacts. Chemical fixatives and polar solvents hamper accurate histochemical demonstration of soluble components of cells and tissues by distortion, displacement, extraction and slow diffusion (Mancini '48). Glycogen is easily soluble in water, therefore aqueous fixatives would not be satisfactory nor reliable.

Gersh in 1932 introduced the Altmann Technique for fixation by drying while freezing. This introduced a method suitable for the accurate localization of substances either normal to the organism, or introduced into the body experimentally. By the use of this technique accurate localization of intravitals with the production of permanent preparation was possible.

With the use of a non-polar solvent (Mancini '48), a histochemical study of glycogen in tissues of the albino mouse was undertaken to determine its distribution qualitatively.

REVIEW OF LITERATURE

Glycogen was discovered by Claude Bernard (1857) in cells of the liver. Other tissues such as the placenta and many embyronic tissues were studied by Bernard (1859), for their glycogen distribution. Plaques of cells in the amniotic membrane of the calf by the use of iodine reaction revealed amorphous and round granules of a brown staining material comparable to cells of the liver. The liver cells contained amorphous and round granules of glycogen.

Schiff said, during the year 1857, that he could identify granules of glycogen in unstained liver. He claimed that these granules were plentiful in livers which contained an abundance of glycogen and scarce in livers containing little glycogen. The glycogen granules were distinguishable from fat droplets and disappeared by amylase digestion. In cartilage and muscle Rouget (1859), stated that the glycogen existed not as granules but as a "plasma". According to Bock and Hoffman (1872), livers containing no glycogen should show granules described by Schiff. Staining with a solution of iodine in potassium iodide a diffuse brown staining network could be seen between colorless granules of Schiff. Salivary digestion would cause the iodine reaction to disappear.

Sections fixed in alcohol (Kyser 1879), (Heidenhain 1883), have the glycogen existing in the form of granules and flakes regularly located in one side of the cell. Other workers including Kulz (1881), pointed out that granularity localized at one end of the cell was an artifact due to the alcohol fixation.

Ehrlich (1883), thought that if granules of glycogen were preformed they should appear in dried preparation. With iodine, a homogenous brown staining of cytoplasm of a dried smear of liver was observed and the nucleus contained no brown color. Lewis (1921) found glycogen in a diffuse condition in cells of Fundulus embryo growing in tissue culture fluid. By using the freezing technique Gersh's ('38) results confirmed those of Ehrilich.

Gierke ('05), observed glistening balls in fresh cartilage cells which corresponded to the location of glycogen upon fixation. He referred to the regularity of glycogen granules in the kidney tubles of the cat as related to glistening balls in the cartilage cells. Arndt ('24), noted a small amount of glycogen deposited extracellularly in the liver. Bartelmez and Bensley ('32), mentioned orientation of glycogen granules in uterine epithelium. They were uncertain whether the granules were preformed or resulted from plasmolysis. In the process of staining by iodine vapor, Lewis ('21), observed that the type of cells in the tissue culture, which showed a diffuse staining previously, would at times suddenly develop blebs or granules of brown staining material upon the death of the cell. Lazarow ('42) stated that iodide solution is a strong protein coagulator and that these changes may well be the result of the iodine coagulator. They do not necessarily account for the vacuolar appearence seen in certain fresh or dried cells.

Kinoshita ('33), reported that the best fixatives before staining tissues to show glycogen were: (1) alcohol-ether containing magnesium sulphate; (2) alcohol-ether made alkaline; and (3) formalin with magnesium sulphate. These gave excellent inflitration. Excellent results were

obtained when alkaline alcohol-ether was injected into the portal vein in situ followed by sectioning of pieces of the liver with the capsule removed.

By using the freezing drying technique and Gersh's apparatus, Mancini ('48) found glycogen diffusely distributed in mammalian liver, cartilage cells, fatty tissue, epithelial cells of the vagina, in the epithelium lining the cavity and the glandular tissue of the uterus, kidney tubules of experimental and human diabetes, in biopsis of human skin from the belly, scrotum, arm and face, in the syncytium of the placenta and in the vesicular cells of the glycogenic organs of the chicken. Good results were obtained with frozen sections. From evidence cited the existence of glycogen as a substance diffusely distributed in cells of the liver is substantiated.

MATERIALS AND METHOD

The albino mice used for this study were kept in a combination wood and screen wire cage in a well ventilated laboratory. The diet consisted of whole grain oats, scratch chicken feed which contained sunflower seeds, corn and wheat, cheese, green cabbage, turnip greens and plenty of fresh water everyday. Every two days a few drops of cod liver oil were placed on the food. Every effort was made to maintain a balanced diet. The cage was brushed and scrubbed daily.

Paraffin and frozen sections of cardiac muscle, liver, kidney and skeletal muscle tissues were used. In preparation of the paraffin sections, the mouse was killed by a blow on the head with a small hammer. The animal was quickly dissected, the tissues dropped into an alcohol-ether fixative saturated with magnesium sulphate (Kinoshita '33) and allowed to remain for twenty-four hours. The tissues were placed in absolute alcohol for two hours after being removed from the fixative. They were then infiltrated

and cleared with cederwood oil in an embedding oven at 56° C. . The tissues remained in the oven until no more bubbles were given off. Paraffin infiltration, embedding and subsequent sectioning followed. Sections were fixed to slides with a mixture of alcohol, fresh egg albumin and a few drops of glycerin. The sections were flattened on the slides by using the alcohol mixture as one would water. They were then allowed to dry and the paraffin was removed by placing the slides in xylol. The histochemical technique involved the use of iodine dissolved in mineral oil, Lilly' M-32 Petronol, (Mancini '44). Sections were placed in a saturated solution of iodine-mineral oil for ten minutes, differentiated for five minutes in an iodine saturated solution of absolute alcohol; washed in absolute alcohol five minutes, and then cleared for ten minutes in oil of thyme. These sections were mounted in Canada balsam.

For the preparation of frozen sections the animal was killed by placing it on a block of dry ice (carbon dioxide) under a small glass which permitted no air to enter. The animal would succumb within one minute or less. Dissection was performed immediately while the animal was still on the ice block. The cardiac muscle, liver, kidney and skeletal muscle tissues were removed and dropped on the ice block where they became frozen through almost instantly, depending on the size of the piece. The temperature of the tissue was below -30° C. after thirty minutes. Each tissue was transferred separately to the plate of a carbon dioxide gas freezing chamber. Here it was kept frozen and embedded in a syrupy solution of gum acacia. The gum acacia syrup was added to the freezing plate around the tissues a little at a time with continued freezing until the tissue was completely covered with a solid frozen mass. Sectioning was done with a Minot Rotary microtome. In

order to get satisfactory freezing on the plate the bottom of the carbon dioxide chamber was raised above the outlet. The best staining method was the same as the above for the paraffin sections.

Other staining methods were used with paraffin and frozen sections. Methods included the use of Ehrlich's haematoxylin and Best's Carmine.

Sections were stained in Ehrlich's haematoxylin for five to ten minutes and blued in tap water substitute made up as follows:

Tap water substitute

Potassium bicarbonate	2 grams
Magnesium sulphate	20 grams
Distilled water up to	1000 C C
total	1000 C C

This solution was saturated with camphor.

The sections were then passed to a two per cent solution of potassium iodide saturated with iodine (a Lugol solution) and left for five minutes. They were removed, tissues wiped around and dehydrated in absolute alcohol saturated in iodine, cleared in thyme oil and mounted in Canada basalm.

In using Best's Carmine stain, sections were stained first in Ehrlich's haematoxylin as for Lugol's solution but differentiated in acid alcohol. After staining for five minutes in Best's Carmine the sections were differentiated in:

Absolute alcohol	80 parts
Methyl alcohol	40 parts
Distilled water	100 parts

until no more red came out (three to five minutes), washed in 80 per cent alcohol and cleared in clove oil. These sections were also mounted in Canada balsam.

Slides were prepared as controls for all tissues studied. These slides had saliva added to them and were set aside until the glycogen was dissolved

by the diastase in the saliva. They were placed in warm water for fifteen minutes or more. The slides were stained as usual by all the methods referred to previously. Comparison between an undigested slide and a digested one assisted in properly identifying the glycogen. (Procedure of the late Dr. B. R. G. Russell, Imperial Cancer Research Laboratory)

RESULTS

By using the carbon dioxide block for special preparation of the frozen sections, the glycogen appeared in a diffuse condition in sections of the liver of the albino mouse. Figure 1 represents the condition that the glycogen assumed in the frozen section. The control which is seen in figure 2 is the result of salivary digestion. Although there is a very small amount of glycogen present in the control, it appears largely as granules in the interstices between the cells.

Paraffin sections of the liver (fig. 3) show granular flocculations of glycogen throughout the tissue. In the control (fig. 4) most of the flocculations have disappeared leaving a diminished amount of scattered glycogen granules. A striking contrast can be seen in diffuse and granulated glycogen by the comparison of figures 1 and 4.

Frozen section of the skeletal muscle (fig. 5) caused the glycogen to appear homogenous in and between the muscle fibers. However, there are areas of varying intensity. The homogeneity might result from the slight amount of contration observed in the striated muscle tissue. In figure 6 salivary digestion has left only a small amount of glycogen granules between the muscle fibers.

In the paraffin section of the skeletal muscle (fig. 7) there is less glycogen present than in the frozen section. Nevertheless, the glycogen

present is preponderantly interfibrillar, appearing as irregular clumps. The control (fig. 8) indicates a disappearance of the glycogen clumps which dominated as seen in figure 7.

In a longitudinal frozen section of kidney tubules,(fig. 9) a large amount of diffuse glycogen appears. There can be seen light lines representing spaces between the tubules. The control (fig. 10) has much less glycogen present. The glycogen present in the control, which is a cross section of the tubules, is not in the intertubular spaces but within the tubules themselves.

Paraffin sections of the kidney (fig. 11) reveal the glycogen as being deposited between the kidney tubules and in the lumen. In the control granulated remnants left after salivary digestion are intratubular (fig, 12).

In frozen sections of the heart (fig. 13) there is a mixture of diffuse and granulated glycogen. Many clearly defined glycogen-free spaces exist in the cardiac tissue. Apparently the heart is well supplied with glycogen but there is not as much present as one would ordinarily assume. The control (fig. 14) reveals a minimum amount of glycogen as compared to the test frozen section. The glycogen in this instance appears somewhat flakey between the muscle fibers.

Glycogen in paraffin sections of cardiac tissue (fig. 15) shows intra and extra fibrous granulation. In the control (fig. 16) a small amount of interfibrous granulated glycogen is present. Frozen sections revealed more glycogen in heart muscle than paraffin sections although the same staining technique was used for both. Frozen and paraffin sections carried through staining methods other than the one outlined using mineral oil as a non-polar solvent were not satisfactory. In staining with Ehrlich's haematoxylin

and subsequently Lugol's solution, nuclei of the cells were outstanding but the glycogen was largely obscure. The haematoxylin apparently masked the true color of the glycogen. This condition might have been due to the presence of artifacts (Mancini '48). The results with haematoxylin and Best's Carmine were not reliable. The sections revealed indication of glycogen stained red but a large amount of the red color was not even in the section intra or extra-cellularly. The Best's Carmine itself produced an unusual amount of artifacts.

Salivary digestion was not one hundred per cent complete in any of the controls studied.

DISCUSSION

A comparison of frozen sections with paraffin sections in a histochemical study of glycogen in tissues of the albino mouse substantiated ideas of granular and diffuse distribution.

Frozen sections stained in a non-polar solvent show more glycogen in liver, skeletal muscle, kidney and cardiac muscle tissues of the white mouse than did the paraffin sections. When diffuse glycogen is seen in paraffin sections there is present a large number of granules. More diffuse glycogen is seen in frozen sections, however, both types of sections reveal some granules. The larger amount of granular glycogen in the paraffin sections is the product of fixation plus the effects produced by heat during preparation.

Glycogen as it appeared in the liver is diffuse in this study, thus giving support to the findings of Ehrlich (1883) Gersh ('38) and Mancini('48). Granulations of glycogen in the paraffin sections of the liver are artifacts. Lazarow ('42) pointed out that the glycogen which appears diffuse in the liver cells is composed of submicroscropic particles. He liberated the particles

from the liver cells by fragmentation. Graphic analysis and multiple correlation by Fern and Lorraine ('40) confirm that glycogen is definitely associated with an appreciable amount of water when it is laid down in the liver. Presumably this applies also to the deposition of glycogen in muscles. McBride, Manson, and Scott ('41' gave evidence to show that the apparent ratio of glycogen to water varies with an increase in the content of non glycogen solids of the liver.

According to observation reported here glycogen in the frozen sections of skeletal muscle appear homogenous in and between the muscle fibers. This observation conflicts with those of some of the other workers. The study of Rojas and Resta's ('38) reveals that glycogen in the muscle fibers was found in the form of small granules in the isotropic disc immediately contiguous to the Z band; no glycogen was found in the band. Gendre ('38) working with fetal and adult muscle from the rat and Guinea pig fixed in Bouin-Allen solution and stained with Lugol aniline blue, said glycogen was in granular concentration in short longitudinal rods in the Q disc of the muscle. Glycogen also appeared external to the muscle fiber in granular form. By using the freezing-drying technique Mancini ('48) demonstrated that glycogen is present only inside the muscle fiber. Explanation for observations reported here of homogenous glycogen in skeletal muscle may be due to the slight amount of contraction observed in the fibers. It is questionable whether granular glycogen changes to homogenous glycogen during contraction.

The method used here for making frozen sections by use of the carbon dioxide block and subsequently staining with iodine in mineral oil reveals much granular and some diffuse glycogen in cardiac tissue. The condition

of the glycogen shown could not have changed appreciably from its original state due to the rapid freezing which began in situ and ended with complete separation of the tissue. Blatherwick et al. ('35), employing largely procedures of other workers concluded that muscles which had been frozen after the hind leg had been severed from the body, contained more lactic acid and less glycogen than muscle that had been frozen in situ. According to Steiner ('35), the freezing of muscle cannot be relied upon to yield absolute values for glycogen. He observed that the contraction elicited by freezing results in a small loss of glycogen, a loss that is reflected in a rise in both fermentable sugar and hexosemonophosphate. To detect this however a chemical analysis would be required. The frozen sections for showing glycogen present in tissues is definitely superior to the paraffin sections.

SUMMARY

1. A comparison of frozen sections with paraffin sections in studying glycogen histochemically in tissues was made.
2. Frozen sections are superior to paraffin sections for staining to show the presence of glycogen.
3. Granular and diffuse glycogen was found in cardiac muscle, skeletal muscle, kidney and liver tissues of the albino mouse.
4. Ideas of granular and diffuse distribution of glycogen in tissues have been substantiated.

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Explanation of Figures

All figures represent tissues of the albinio mouse stained in a saturated solution of iodine-mineral oil. Digested sections are paired with undigested sections. Frozen sections are compared with paraffin sections for glycogen content.

Figure 1. Liver Frozen Section Undigested

Figure 2. Liver Frozen Section Digested

Figure 3. Liver Paraffin Section Undigested

Figure 4. Liver Paraffin Digested

Figure 5. Skeletal Muscle Frozen Section Undigested

Figure 6. Skeletal Muscle Frozen Section Digested

Figure 7. Skeletal Muscle Paraffin Section Undigested

Figure 8. Skeletal Muscle Paraffin Section Digested

Figure 9. Kidney Frozen Section Undigested

Figure 10. Kidney Frozen Section Digested

Figure 11. Kidney Paraffin Section Undigested

Figure 12. Kidney Paraffin Section Digested

Figure 13. Cardiac Muscle Frozen Section Undigested

Figure 14. Cardiac Muscle Frozen Section Digested

Figure 15. Cardiac Muscle Paraffin Section Undigested

Figure 16. Cardiac Muscle Paraffin Section Digested

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